



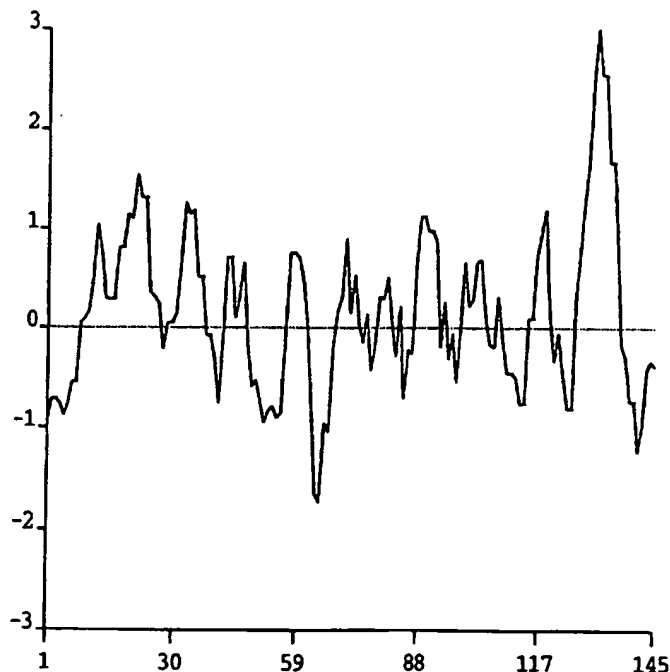
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/12, C07K 14/47, C12N 15/70, I/21, C07K 16/18, A61K 38/17		A1	(11) International Publication Number: WO 98/06847
			(43) International Publication Date: 19 February 1998 (19.02.98)
(21) International Application Number: PCT/US97/14379		(81) Designated States: AT, AU, BR, CA, CH, CN, DE, DK, ES, FI, GB, IL, JP, KR, MX, NO, NZ, RU, SE, SG, US, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 14 August 1997 (14.08.97)		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 08/700,626 16 August 1996 (16.08.96) US			
(60) Parent Application or Grant (63) Related by Continuation US 08/700,626 (CIP) Filed on 16 August 1996 (16.08.96)			
(71) Applicant (for all designated States except US): INCYTE PHARMACEUTICALS, INC. [US/US]; 3174 Porter Drive, Palo Alto, CA 94304 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): AU-YOUNG, Janice [US/US]; 1419 Kains Avenue, Berkeley, CA 94702 (US). HILLMAN, Jennifer, L. [US/US]; 230 Monroe Drive #12, Mountain View, CA 94040 (US). GOLI, Surya, K. [IN/US]; 620 Iris Avenue #338, Sunnyvale, CA 94086 (US).			
(74) Agent: BILLINGS, Lucy, J.; Incyte Pharmaceuticals, Inc., 3174 Porter Drive, Palo Alto, CA 94304 (US).			

(54) Title: HUMAN DBI/ACBP-LIKE PROTEIN

(57) Abstract

The present invention provides polynucleotides which identify and encode a human Diazepam binding inhibitor/acyl-CoA binding protein (DBI/ACBP)-like protein (DBIH). The invention provides for genetically engineered expression vectors and host cells comprising the nucleic acid sequence encoding DBIH. The invention also provides for the use of substantially purified DBIH for drug delivery as well as for the production of recombinant proteins for the treatment of diseases associated with the expression of DBIH. Additionally, the invention provides for the use of antisense molecules to DBIH in the treatment of diseases associated with the expression of DBIH. The invention also describes diagnostic assays which utilize diagnostic compositions comprising the polynucleotides which hybridize with naturally occurring sequences encoding DBIH and antibodies which specifically bind to the protein.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

HUMAN DBI/ACBP-LIKE PROTEIN

TECHNICAL FIELD

The present invention relates to nucleic acid and amino acid sequences of a novel human DBI/ACBP-like protein and to the use of these sequences in the diagnosis, study, prevention and
5 treatment of disease.

BACKGROUND ART

Diazepam binding inhibitor/acyl-CoA binding protein (DBI/ACBP)-like protein is a 10 kdal protein found in species ranging from yeast to mammals. It is expressed in a variety of organs and tissues. Originally, DBI was purified from rat brain based on its ability to displace
10 diazepam from type A gamma-aminobutyrate (GABA_A) receptors (Guidotti et al (1983) Proc Nat Acad Sci USA 80:3531-3535). An acyl-Coenzyme A (acyl-CoA) binding protein (ACBP) subsequently purified from liver was found to be identical to DBI (Mikkelsen J et al (1987) Biochem J 245:857-861). The protein was known as endozepine, DBI, or ACBP, but it is now generally referred to as DBI/ACBP. DBI/ACBP, and polypeptides derived from it, have been
15 implicated in multiple biological processes, such as 1) GABA_A/benzodiazepam receptor modulation, 2) acyl-CoA metabolism, 3) steroidogenesis, and 4) insulin secretion (reviewed in Knudsen J et al (1993) Mol Cell Biochem 123:129-138).

The three-dimensional solution structure of bovine DBI/ACBP with and without bound acyl-CoA ligands has been solved by NMR (Andersen KV and Poulsen FM (1992) J Mol Biol
20 226:1131-41; Kragelund et al (1993) J Mol Biol 230:1260-1277). DBI/ACBP consists of four alpha helices (A1 through A4) arranged in a left-handed anti-parallel bundle, with parallel helices A1 and A4 anti-parallel to helices A2 and A3. Helix A2 interacts with each of the other three helices in a structure reminiscent of a bowl. The inner surface of the bowl has a patch of non-polar and uncharged residues at the interface between helices A2 and A3. The rims of the bowl
25 have mainly polar and charged groups which are contributed by the hydrophilic residues of the amphipathic helices. The ligand binding site is located on the inner surface of the bowl, and it binds the aliphatic acyl chain of the fatty acyl-CoA ligand in a non-polar arrangement created partly by the protein and partly by the pantetheine and the adenosine-3'-phosphate of CoA. The pantetheine and CoA moieties likewise form a highly polar and charged surface, so that the
30 surface together with the polar and charged rims of the protein bowl ensure the solubility of the entire complex (Kragelund et al (1993) J Mol Biol 230:1260-1277).

The binding affinity of bovine DBI/ACBP towards acyl-CoA esters depends on the length

of the acyl chain, where the highest affinity is for long-chain (C14 to C22) acyl-CoA esters. The protein is very specific in binding acyl-CoA esters, binding neither free CoA nor free fatty acids (Rosendal J et al (1993) *Biochem J* 290:321-326). DBI/ACBP sequesters bound long-chain fatty acyl-CoA, protects acyl-CoAs from hydrolysis, extracts acyl-CoAs from phosphatidyl choline
5 membranes, and mediates intermembrane acyl-CoA transport (Rasmussen et al (1994) *Biochem J* 299:165-170). The overexpression of DBI/ACBP in rapidly growing brain tumors such as astrocytomas, glioblastomas and medullablastomas suggests that it may be involved in the regulation of high-energy acyl-CoA metabolism in rapidly growing neuronal cells (Alhi H et al (1995) *Cell Growth Differ* 6:309-314).

- 10 DBI/ACBP also inhibits the binding of benzodiazepines to the GABA_A receptor. The GABA_A receptor is a post-synaptic Cl⁻ channel. The Cl⁻ ion channel opening burst, elicited by the inhibitory neurotransmitter GABA, is prolonged by benzodiazepines. Benzodiazepines thereby enhance GABA-mediated synaptic inhibitory responses and reduce pathological anxiety. DBI/ACBP or its proteolytic fragments, most notably octadecaneneuropeptide (ODN,
15 DBI/ACBP amino acids 32-50), suppress the anxiety-reducing effect of the benzodiazepines. Expression of DBI/ACBP is increased in brain and cerebrospinal fluid of patients diagnosed with neurological disorders such as hepatic encephalopathy, depression and anxiety (Costa E and Guidotti A (1991) *Life Sciences* 49:325-344).

- DBI/ACBP is also involved in the regulation of steroid biosynthesis in mitochondria
20 (Garnier et al (1993) *Endocrinology* 132:444-458). DBI/ACBP stimulates mitochondrial steroidogenesis in the adrenal gland by facilitating cholesterol delivery to the inner mitochondrial membrane (Yanagibashi K (1988) *Endocrinology* 123:2075-2082). Knudsen et al (1993, supra) suggest that DBI/ACBP may also scavenge fatty acyl-CoA esters produced from fatty acids released in the conversion of cholesterol to steroids. Antisense oligonucleotides to DBI/ACBP
25 inhibit hormone-stimulated steroid production in Leydig cells of rat testis (Boujrad N et al (1993) *Proc Nat Acad Sci USA* 90:5728-5731).

- DBI/ACBP has been found in all tissues tested; the highest amounts have been found in liver, kidney, brain, adrenal gland, intestine and salivary gland (Knudsen J et al (1993) *Mol Cell Biochem* 123:129-138). Immunohistochemical localization indicates DBI/ACBP is selectively
30 expressed in specialized cells within a given organ. Elevated levels of DBI/ACBP have been found in cells of the adrenal cortex and testis which produce steroids, and in liver hepatocytes which are involved in steroid and fat metabolism. Elevated levels of DBI/ACBP have also been

found in epithelial cells of kidney tubules, the upper intestinal tracts and large bronchioles, cells which are specialized for water and electrolyte absorption and secretion. In brain high DBI/ACBP concentrations are found in choroid plexus and circumventricular organs, which are specialized for the control of secretion and osmolality of cerebrospinal fluid (Bovolin et al (1990)

5 Reg Peptides 29:267-281).

The selective modulation of the expression or activity of a novel tissue-specific DBI/ACBP-like protein may allow the successful management of diseases or biochemical abnormalities relating to the tissues in which it is expressed. In addition, the binding properties of this small protein may be utilized in drug delivery applications as a soluble carrier for
10 otherwise insoluble therapeutic molecules.

DISCLOSURE OF THE INVENTION

The present invention discloses a novel tissue-specific DBI/ACBP-like protein, hereinafter referred to as DBIH, having chemical and structural homology to isoforms of human DBI/ACBP and bovine DBI/ACBP. Accordingly, the invention features a substantially purified
15 DBI/ACBP-like protein, encoded by amino acid sequence of SEQ ID NO:1, having structural characteristics of the family of DBI/ACBPs including those from human and cow.

One aspect of the invention features isolated and substantially purified polynucleotides which encode DBIH. In a particular aspect, the polynucleotide is the nucleotide sequence of SEQ ID NO:2. In addition, the invention features nucleotide sequences which hybridize under
20 stringent conditions to SEQ ID NO:2.

The invention further relates to nucleic acid sequence encoding DBIH, oligonucleotides, peptide nucleic acids (PNA), fragments, portions or antisense molecules thereof. The present invention also relates to an expression vector which includes polynucleotide encoding DBIH and its use to transform host cells or organisms. The invention also relates to antibodies which bind
25 specifically to the DBI of SEQ ID NO:1 and to a pharmaceutical composition comprising a substantially purified DBI of SEQ ID NO:1.

BRIEF DESCRIPTION OF DRAWINGS

Figures 1A, 1B and 1C show the amino acid sequence (SEQ ID NO:1) and the nucleic acid sequence (SEQ ID NO:2) of the human DBI/ACBP-like protein DBIH, produced using
30 MacDNAsis software (Hitachi Software Engineering Co Ltd, San Bruno CA).

Figures 2A and 2B shows the amino acid sequence alignments among DBIH (SEQ ID NO:1), the 104 amino acid human DBI/ACBP isoform (GI 181478; SEQ ID NO:3), the 86 amino

acid human DBI/ACBP isoform (GI 118276; SEQ ID NO:4), and the 86 amino acid bovine DBI/ACBP (GI 118275, SEQ ID NO:5), produced using the multisequence alignment program of DNASTar software (DNASTar Inc, Madison WI).

Figure 3 shows the hydrophobicity plot (generated using MacDNAsis software) for DBIH, SEQ ID NO:1; the X axis reflects amino acid position, and the negative Y axis, hydrophobicity.

Figure 4 shows the predicted secondary structure (generated using MacDNAsis software) of DBIH, SEQ ID NO:1.

MODES FOR CARRYING OUT THE INVENTION

10 **Definitions**

"Nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand.

Similarly, amino acid sequence as used herein refers to peptide or protein sequence.

15 "Peptide nucleic acid" as used herein refers to a molecule which comprises an oligomer to which an amino acid residue, such as lysine, and an amino group have been added. These small molecules, also designated anti-gene agents, stop transcript elongation by binding to their complementary (template) strand of nucleic acid (Nielsen PE et al (1993) Anticancer Drug Des 8:53-63).

20 A "variant" of DBIH is defined as an amino acid sequence that is different by one or more amino acid substitutions. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, eg, replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, eg, replacement of a glycine with a tryptophan. Similar minor variations may also include amino acid deletions or
25 insertions, or both. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTar software.

A "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

30 An "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring DBIH.

A "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

The term "biologically active" refers to a DBIH having structural, regulatory or biochemical functions of the naturally occurring DBIH. Likewise, "immunologically active" defines the capability of the natural, recombinant or synthetic DBIH, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The term "derivative" as used herein refers to the chemical modification of a nucleic acid encoding DBIH or the encoded DBIH. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative would encode a polypeptide which retains essential biological characteristics of natural DBIH.

As used herein, the term "substantially purified" refers to molecules, either nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated.

"Stringency" typically occurs in a range from about $T_m - 5^\circ\text{C}$ (5°C below the T_m of the probe) to about 20°C to 25°C below T_m . As will be understood by those of skill in the art, a stringency hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences.

The term "hybridization" as used herein shall include "any process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York NY). Amplification as carried out in the polymerase chain reaction technologies is described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY).

25 Description

The present invention relates to a novel human DBI/ACBP-like protein, designated DBIH, initially identified among the partial cDNAs from a human paraganglia tissue library (PGANNOT01) and to the use of the nucleic acid and amino acid sequences disclosed herein in the study, diagnosis, prevention and treatment of disease. Northern analysis using the LIFESEQ™ database (Incyte Pharmaceuticals, Palo Alto CA) indicates that DBIH-encoding mRNA is only present in paraganglia, in contrast to the wide tissue distribution of the DBI/ACBPs. Paraganglia contain cells that synthesize, store and secrete catecholamines. Cells of

the paraganglia receive sympathetic preganglionic innervation similar to that of chromaffin cells of the adrenal medulla. The paraganglia are well vascularized and the secretory cells are generally located next to capillaries. With little obstruction to the passage of hormones, paraganglial cells have both remote and local endocrine effects.

5 The present invention also encompasses DBIH variants. A preferred DBIH variant is one having at least 80% amino acid sequence similarity to the DBIH amino acid sequence (SEQ ID NO:1), a more preferred DBIH variant is one having at least 90% amino acid sequence similarity to SEQ ID NO:1 and a most preferred DBIH variant is one having at least 95% amino acid sequence similarity to SEQ ID NO:1.

10 The nucleic acid sequence encoding a portion of DBIH was first identified in the cDNA, Incyte Clone 620984, through a computer-generated search for amino acid sequence alignments. The nucleic acid sequence, SEQ ID NO:2, disclosed herein (Figures 1A, 1B and 1C) encodes the amino acid sequence, SEQ ID NO:1, designated DBIH. The present invention is based in part on the structural homology shown in Figs 2A and 2B, among DBIH and other DBI/ACBPs
15 including two human isoforms (GI 181478; Gray PW et al (1986) Proc Nat Acad Sci USA 83:7547-7551 and GI 118276; Marquardt H et al (1986) J Biol Chem 261:9727-9731), and one bovine isoform (GI 118275, Marquardt H et al, supra). GI 181478, GI 118276, and GI 118275 have, respectively, 39%, 42% and 40% sequence identity with DIBH.

DBIH consists of 145 amino acids and, based on the hydropathy plot (Figure 3) and the
20 secondary structure prediction (Figure 4), is a soluble protein consisting of at least four helical segments. From its homology to the central portion of human and bovine DBI/ACBPs, the four alpha-helices of DBIH are predicted to include residues at or near positions 45-56, 61-77, 92-103, and 106-121. In comparison with the 86 residue human and bovine DBI/ACBPs (GI 118276 and GI 118275, respectively), DBIH contains an additional 40 amino acids at the N-terminus and an
25 additional 14 amino acids near the C-terminus (Figures 2A and 2B). The 104 amino acid human DBI/ACBP isoform (GI 181478) likewise contains an additional 23 amino acids near the N-terminus compared to the 86 residue forms. The additional residues in DBIH at the N- and C-termini do not have significant alpha-helical content (Figure 4), and contain a large proportion of hydrophilic charged residues, indicating they are not likely to be membrane-spanning or signal
30 sequences. However, the additional residues at the N- or C- termini of DBIH may be proteolytically processed to yield smaller forms of DBIH in vivo.

THE DBIH CODING SEQUENCES

The nucleic acid and amino acid sequences of DBIH are shown in Figures 1A, 1b and 1c. In accordance with the invention, any nucleic acid sequence which encodes the amino acid sequence of DBIH can be used to generate recombinant molecules which express DBIH. In a
5 specific embodiment described herein, a partial sequence of DBIH was first isolated as Incyte Clone 620984 from a human paraganglia tissue library (PGANNOT01).

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of DBIH-encoding nucleotide sequences, some bearing minimal homology to the nucleotide sequences of any known and naturally occurring gene may be
10 produced. The invention contemplates each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring DBIH, and all such variations are to be considered as being specifically disclosed.

15 Although nucleotide sequences which encode DBIH and its variants are preferably capable of hybridizing to the nucleotide sequence of the naturally occurring DBIH under appropriately selected conditions of stringency, it may be advantageous to produce nucleotide sequences encoding DBIH or its derivatives possessing a substantially different codon usage. Codons may be selected to increase the rate at which expression of the peptide occurs in a
20 particular prokaryotic or eukaryotic expression host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding DBIH and its derivatives without altering the encoded amino acid sequences include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

25 It is now possible to produce a DNA sequence, or portions thereof, encoding a DBIH and its derivatives entirely by synthetic chemistry, after which the synthetic gene may be inserted into any of the many available DNA vectors and cell systems using reagents that are well known in the art at the time of the filing of this application. Moreover, synthetic chemistry may be used to introduce mutations into a gene encoding DBIH.

30 Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridizing to the nucleotide sequence of Figures 1A, 1B and 1C under various conditions of stringency. Hybridization conditions are based on the melting temperature (T_m) of

the nucleic acid binding complex or probe, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA) incorporated herein by reference, and confer may be used at a defined stringency.

Altered nucleic acid sequences encoding DBIH which may be used in accordance with the invention include deletions, insertions or substitutions of different nucleotides resulting in a polynucleotide that encodes the same or a functionally equivalent DBIH. The protein may also show deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent DBIH. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the biological activity of DBIH is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine phenylalanine, and tyrosine.

Included within the scope of the present invention are alleles of DBIH. As used herein, an "allele" or "allelic sequence" is an alternative form of DBIH. Alleles result from a mutation, ie, a change in the nucleic acid sequence, and generally produce altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given gene may have none, one or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to natural deletions, additions or substitutions of amino acids. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

Methods for DNA sequencing are well known in the art and employ such enzymes as the Klenow fragment of DNA polymerase I, Sequenase® (US Biochemical Corp, Cleveland OH), Taq polymerase (Perkin Elmer, Norwalk CT), thermostable T7 polymerase (Amersham, Chicago IL), or combinations of recombinant polymerases and proofreading exonucleases such as the ELONGASE Amplification System marketed by Gibco BRL (Gaithersburg MD). Preferably, the process is automated with machines such as the Hamilton Micro Lab 2200 (Hamilton, Reno NV), Peltier Thermal Cycler (PTC200; MJ Research, Watertown MA) and the ABI 377 DNA sequencers (Perkin Elmer).

EXTENDING THE POLYNUCLEOTIDE SEQUENCE

The polynucleotide sequence encoding DBIH may be extended utilizing partial nucleotide

sequence and various methods known in the art to detect upstream sequences such as promoters and regulatory elements. Gobinda et al (1993; PCR Methods Applic 2:318-22) disclose "restriction-site" polymerase chain reaction (PCR) as a direct method which uses universal primers to retrieve unknown sequence adjacent to a known locus. First, genomic DNA is
5 amplified in the presence of primer to a linker sequence and a primer specific to the known region. The amplified sequences are subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR can be used to amplify or extend sequences using divergent primers based
10 on a known region (Triglia T et al (1988) Nucleic Acids Res 16:8186). The primers may be designed using OLIGO® 4.06 Primer Analysis Software (1992; National Biosciences Inc, Plymouth MN), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72° C. The method uses several restriction enzymes to generate a suitable fragment in the known region
15 of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Capture PCR (Lagerstrom M et al (1991) PCR Methods Applic 1:111-19) is a method for PCR amplification of DNA fragments adjacent to a known sequence in human and yeast artificial chromosome DNA. Capture PCR also requires multiple restriction enzyme digestions and
20 ligations to place an engineered double-stranded sequence into an unknown portion of the DNA molecule before PCR.

Another method which may be used to retrieve unknown sequences is that of Parker JD et al (1991; Nucleic Acids Res 19:3055-60). Additionally, one can use PCR, nested primers and PromoterFinder libraries to walk in genomic DNA (PromoterFinder™ Clontech (Palo Alto CA).
25 This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

Preferred libraries for screening for full length cDNAs are ones that have been size-selected to include larger cDNAs. Also, random primed libraries are preferred in that they will contain more sequences which contain the 5' and upstream regions of genes. A randomly primed library may be particularly useful if an oligo d(T) library does not yield a full-length
30 cDNA. Genomic libraries are useful for extension into the 5' nontranslated regulatory region.

Capillary electrophoresis may be used to analyze the size or confirm the nucleotide sequence of sequencing or PCR products. Systems for rapid sequencing are available from

Perkin Elmer, Beckman Instruments (Fullerton CA), and other companies. Capillary sequencing may employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity is converted to electrical signal using appropriate software (eg. Genotyper™ and Sequence Navigator™ from Perkin Elmer) and the entire process from loading of samples to computer analysis and electronic data display is computer controlled. Capillary electrophoresis is particularly suited to the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample. The reproducible sequencing of up to 350 bp of M13 phage DNA in 30 min has been reported

10 (Ruiz-Martinez MC et al (1993) Anal Chem 65:2851-8).

EXPRESSION OF THE NUCLEOTIDE SEQUENCE

In accordance with the present invention, polynucleotide sequences which encode DBIH, fragments of the polypeptide, fusion proteins or functional equivalents thereof may be used in recombinant DNA molecules that direct the expression of DBIH in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used to clone and express DBIH. As will be understood by those of skill in the art, it may be advantageous to produce DBIH-encoding nucleotide sequences possessing non-naturally occurring codons. Codons preferred by a particular prokaryotic or eukaryotic host (Murray E et al (1989) Nuc Acids Res

15 17:477-508) can be selected, for example, to increase the rate of DBIH expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

The nucleotide sequences of the present invention can be engineered in order to alter a coding sequence of DBIH for a variety of reasons, including but not limited to, alterations which modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, eg, site-directed mutagenesis to insert new restriction sites, to alter glycosylation patterns, to change codon preference, to produce splice variants, etc.

25

In another embodiment of the invention, a natural, modified or recombinant nucleotide sequence encoding DBIH may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for inhibitors of DBIH activity, it may be useful to encode a chimeric DBIH protein that is recognized by a commercially available antibody. A

30

fusion protein may also be engineered to contain a cleavage site located between a DBIH sequence and the heterologous protein sequence, so that the DBIH may be cleaved and substantially purified away from the heterologous moiety.

In an alternate embodiment of the invention, the coding sequence for DBIH may be synthesized, whole or in part, using chemical methods well known in the art (see Caruthers MH et al (1980) Nuc Acids Res Symp Ser 215-23, Horn T et al (1980) Nuc Acids Res Symp Ser 225-32. etc). Alternatively, the protein itself could be produced using chemical methods to synthesize a DBIH amino acid sequence, whole or in part. For example, peptide synthesis can be performed using various solid-phase techniques (Roberge JY et al (1995) Science 269:202-204) and automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The newly synthesized peptide can be substantially purified by preparative high performance liquid chromatography (eg, Creighton (1983) Proteins, Structures and Molecular Principles, WH Freeman and Co, New York NY). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (eg, the Edman degradation procedure; Creighton, supra). Additionally the amino acid sequence of DBIH, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

EXPRESSION SYSTEMS

In order to express a biologically active DBIH, the nucleotide sequence encoding DBIH or its functional equivalent, is inserted into an appropriate expression vector, ie, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing a DBIH coding sequence and appropriate transcriptional or translational controls. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination or genetic recombination. Such techniques are described in Sambrook et al (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview NY and Ausubel FM et al (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York NY.

A variety of expression vector/host systems may be utilized to contain and express a DBIH coding sequence. These include but are not limited to microorganisms such as bacteria

transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (eg, baculovirus); plant cell systems transfected with virus expression vectors (eg, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with bacterial
5 expression vectors (eg, Ti or pBR322 plasmid); or animal cell systems.

The "control elements" or "regulatory sequences" of these systems vary in their strength and specificities and are those nontranslated regions of the vector, enhancers, promoters, and 3' untranslated regions, which interact with host cellular proteins to carry out transcription and translation. Depending on the vector system and host utilized, any number of suitable
10 transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the Bluescript® phagemid (Stratagene, LaJolla CA) or pSport1 (Gibco BRL) and ptrp-lac hybrids and the like may be used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (eg, heat
15 shock, RUBISCO; and storage protein genes) or from plant viruses (eg, viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from the mammalian genes or from mammalian viruses are most appropriate. If it is necessary to generate a cell line that contains multiple copies of DBIH, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

20 In bacterial systems, a number of expression vectors may be selected depending upon the use intended for DBIH. For example, when large quantities of DBIH are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be desirable. Such vectors include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors such as Bluescript® (Stratagene), in which
25 the DBIH coding sequence may be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of β -galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke & Schuster (1989) J Biol Chem 264:5503-5509); and the like. pGEX vectors (Promega, Madison WI) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble
30 and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems are designed to include heparin, thrombin or factor XA protease cleavage sites so that the cloned polypeptide of

interest can be released from the GST moiety at will.

In the yeast, Saccharomyces cerevisiae, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase and PGH may be used. For reviews, see Ausubel et al (supra) and Grant et al (1987) *Methods in Enzymology* 153:516-544.

5 In cases where plant expression vectors are used, the expression of a sequence encoding DBIH may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV (Brisson et al (1984) *Nature* 310:511-514) may be used alone or in combination with the omega leader sequence from TMV (Takamatsu et al (1987) *EMBO J* 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al
10 (1984) *EMBO J* 3:1671-1680; Broglie et al (1984) *Science* 224:838-843); or heat shock promoters (Winter J and Sinibaldi RM (1991) *Results Probl Cell Differ* 17:85-105) may be used. These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. For reviews of such techniques, see Hobbs S or Murry LE in McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill New York NY, pp
15 191-196 or Weissbach and Weissbach (1988) Methods for Plant Molecular Biology, Academic Press, New York NY, pp 421-463.

An alternative expression system which could be used to express DBIH is an insect system. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in Spodoptera frugiperda cells or in Trichoplusia larvae. The
20 DBIH coding sequence may be cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of the DBIH coding sequence will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein coat. The recombinant viruses are then used to infect S. frugiperda cells or Trichoplusia larvae in which DBIH is expressed (Smith et al (1983) *J Virol* 46:584;
25 Engelhard EK et al (1994) *Proc Nat Acad Sci* 91:3224-7).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, a coding sequence for DBIH may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a nonessential E1 or E3 region of the viral genome will
30 result in a viable virus capable of expressing DBIH in infected host cells (Logan and Shenk (1984) *Proc Natl Acad Sci* 81:3655-59). In addition, transcription enhancers, such as the rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Specific initiation signals may also be required for efficient translation of a DBIH sequence. These signals include the ATG initiation codon and adjacent sequences. In cases where nucleic acid encoding DBIH, its initiation codon and upstream sequences are inserted into the appropriate expression vector, no additional translational control signals may be needed.

5 However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous transcriptional control signals including the ATG initiation codon must be provided.

Furthermore, the initiation codon must be in the correct reading frame to ensure transcription of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the

10 inclusion of enhancers appropriate to the cell system in use (Scharf D et al (1994) Results Probl Cell Differ 20:125-62; Bittner et al (1987) Methods in Enzymol 153:516-544).

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation,

15 glycosylation, phosphorylation, lipidation and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be important for correct insertion, folding and/or function. Different host cells such as CHO, HeLa, MDCK, 293, WI38, etc have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the introduced, foreign protein.

20 For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express DBIH may be transformed using expression vectors which contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose
25 of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clumps of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These
30 include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler M et al (1977) Cell 11:223-32) and adenine phosphoribosyltransferase (Lowy I et al (1980) Cell 22:817-23) genes which can be employed in tk- or aprt- cells, respectively. Also, antimetabolite, antibiotic

or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler M et al (1980) Proc Natl Acad Sci 77:3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin F et al (1981) J Mol Biol 150:1-14) and als or pat, which confer resistance to chlorsulfuron and phosphinotricin
5 acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman SC and RC Mulligan (1988) Proc Natl Acad Sci 85:8047-51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins, β glucuronidase and its substrate, GUS, and luciferase and its substrate,
10 luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes CA et al (1995) Methods Mol Biol 55:121-131).

IDENTIFICATION OF TRANSFORMANTS CONTAINING THE POLYNUCLEOTIDE SEQUENCE

15 Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression should be confirmed. For example, if the DBIH polynucleotide sequence is inserted within a marker gene sequence, recombinant cells containing DBIH can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a DBIH sequence under the control of a single
20 promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem DBIH as well.

Alternatively, host cells which contain the coding sequence for DBIH and express DBIH may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or
25 immunoassay techniques which include membrane, solution, or chip based technologies for the detection and/or quantification of the nucleic acid or protein.

The presence of the polynucleotide sequence encoding DBIH can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes, portions or fragments of DBIH-encoding nucleotides. Nucleic acid amplification based assays involve the use of
30 oligonucleotides or oligomers based on the DBIH sequence to detect transformants containing DBIH DNA or RNA. As used herein "oligonucleotides" or "oligomers" refer to a nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides, preferably about

15 to 30 nucleotides, and more preferably about 20-25 nucleotides which can be used as a probe or amplimer.

A variety of protocols for detecting and measuring the expression of DBIH, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples
5 include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on DBIH is preferred, but a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton R et al (1990, Serological Methods, a Laboratory Manual, APS Press, St Paul MN) and
10 Maddox DE et al (1983, J Exp Med 158:1211).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to DBIH include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the
15 DBIH sequence, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides.

A number of companies such as Pharmacia Biotech (Piscataway NJ), Promega (Madison
20 WI), and US Biochemical Corp (Cleveland OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US Patents 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241. Also,
25 recombinant immunoglobulins may be produced as shown in US Patent No. 4,816,567 incorporated herein by reference.

PURIFICATION OF DBIH

Host cells transformed with a DBIH-encoding nucleotide sequence may be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The
30 protein produced by a recombinant cell may be contained intracellularly or secreted depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing DBIH can be designed for efficient production and proper transmembrane

insertion of DBIH into a prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join DBIH to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll DJ et al (1993) DNA Cell Biol 12:441-53; cf discussion of vectors infra containing fusion proteins).

- 5 DBIH may also be expressed as a recombinant protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity
- 10 purification system (Immunex Corp, Seattle WA). The inclusion of a cleavable linker sequences such as Factor XA or enterokinase (Invitrogen, San Diego CA) between the purification domain and DBIH is useful to facilitate purification. One such expression vector provides for expression of a fusion protein comprising an DBIH and contains nucleic acid encoding 6 histidine residues followed by thioredoxin and an enterokinase cleavage site. The histidine residues
- 15 facilitate purification on IMIAC (immobilized metal ion affinity chromatography as described in Porath et al (1992) Protein Expression and Purification 3:263-281) while the enterokinase cleavage site provides a means for purifying the DBI from the fusion protein.

- In addition to recombinant production, fragments of DBIH may be produced by direct peptide synthesis using solid-phase techniques (cf Stewart et al (1969) Solid-Phase Peptide
- 20 Synthesis, WH Freeman Co, San Francisco; Merrifield J (1963) J Am Chem Soc 85:2149-2154). In vitro protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City CA) in accordance with the instructions provided by the manufacturer. Various fragments of DBIH may be chemically synthesized separately and
- 25 combined using chemical methods to produce the full length molecule.

USES OF DBIH

- The rationale for the use of polynucleotide and polypeptide sequences disclosed herein is based in part on the chemical and structural homology among the novel DBIH and the human and bovine isoforms of DBI/ACBP. DBIH may be used in the diagnosis and treatment of conditions,
- 30 disorders or diseases associated with abnormal function of paraganglia, including paragangliomas.

The clinical features and morbidity of paragangliomas are due predominantly to the

abnormal release of catecholamines. Hypertension is the most common manifestation. Paraganglioma is a correctable cause of high blood pressure. Indeed, it is rarely fatal if properly diagnosed and treated.

DBIH may be useful in the regulation of the biosynthesis or metabolism of biological molecules such as catecholamines in paraganglia. Molecules associated with paraganglial function, or their precursors or metabolic products, may bind to DBIH in a manner analogous to that of fatty acyl-CoAs to DBI/ACBP. DBIH may sequester and protect the bound ligand from unwanted side-reactions such as hydrolysis or oxidation, or present the bound ligand to a receptor molecule. Alternatively, DBIH or a fragment thereof may act as a neuromodulator of catecholamine-induced responses in paraganglia.

DBIH or its fragments can be used to identify specific molecules which it sequesters or with which it interacts. In this regard, DBIH may also be used to sequester therapeutic agents, specifically binding the drug so that the complex is water-soluble and suitable for therapeutic delivery.

In overexpression of DBIH associated with paraganglia-related disorders, it may be advantageous to suppress DBIH. DBIH could be suppressed by administration of antisense oligonucleotides. Alternatively, antibodies specifically recognizing the active site of DBIH may be introduced to treat diseases or conditions associated with abnormal DBIH activity.

The structure of DBIH also allows its use in delivery of other therapeutic molecules. DBIH may bind therapeutic agents or drugs which are ordinarily insoluble or only slightly soluble in water. The high charge density of the DBIH molecule renders the protein-ligand complex soluble in an aqueous environment. The specificity and binding affinities of DBIH for therapeutic ligands may be manipulated by protein engineering techniques known to those skilled in the art.

DBIH ANTIBODIES

DBIH-specific antibodies are useful for the diagnosis of conditions and diseases associated with expression of DBIH. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single-chain, Fab fragments and fragments produced by a Fab expression library. Neutralizing antibodies, ie, those which inhibit dimer formation, are especially preferred for diagnostics and therapeutics.

DBIH for antibody induction does not require biological activity; however, the protein fragment, or oligopeptide must be antigenic. Peptides used to induce specific antibodies may

have an amino acid sequence consisting of at least five amino acids, preferably at least 10 amino acids. Preferably, they should mimic a portion of the amino acid sequence of the natural protein and may contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of DBIH amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule. Procedures well known in the art can be used for the production of antibodies to DBIH.

For the production of antibodies, various hosts including goats, rabbits, rats, mice, etc may be immunized by injection with DBIH or any portion, fragment or oligopeptide which retains immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are potentially useful human adjuvants.

Monoclonal antibodies to DBIH may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Koehler and Milstein (1975 Nature 256:495-497), the human B-cell hybridoma technique (Kosbor et al (1983) Immunol Today 4:72; Cote et al (1983) Proc Natl Acad Sci 80:2026-2030) and the EBV-hybridoma technique (Cole et al (1985) Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, New York NY, pp 77-96).

In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison et al (1984) Proc Natl Acad Sci 81:6851-6855; Neuberger et al (1984) Nature 312:604-608; Takeda et al (1985) Nature 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US Patent No. 4,946,778) can be adapted to produce DBIH-specific single chain antibodies

Antibodies may also be produced by inducing in vivo production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi et al (1989, Proc Natl Acad Sci 86:3833-3837), and Winter G and Milstein C (1991; Nature 349:293-299).

Antibody fragments which contain specific binding sites for DBIH may also be generated.

For example, such fragments include, but are not limited to, the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab
5 fragments with the desired specificity (Huse WD et al (1989) Science 256:1275-1281).

A variety of protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the formation of complexes between DBIH and its specific antibody and the measurement of complex formation. A two-site, monoclonal-based
10 immunoassay utilizing monoclonal antibodies reactive to two noninterfering epitopes on a specific DBIH protein is preferred, but a competitive binding assay may also be employed. These assays are described in Maddox DE et al (1983, J Exp Med 158:1211).

DIAGNOSTIC ASSAYS USING DBIH SPECIFIC ANTIBODIES

Particular DBIH antibodies are useful for the diagnosis of conditions or diseases
15 characterized by expression of DBIH or in assays to monitor patients being treated with DBIH, agonists or inhibitors. Diagnostic assays for DBIH include methods utilizing the antibody and a label to detect DBIH in human body fluids or extracts of cells or tissues. The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, the polypeptides and antibodies will be labeled by joining them, either covalently or noncovalently,
20 with a reporter molecule. A wide variety of reporter molecules are known, several of which were described above.

A variety of protocols for measuring DBIH, using either polyclonal or monoclonal antibodies specific for the respective protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent
25 activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on DBIH is preferred, but a competitive binding assay may be employed. These assays are described, among other places, in Maddox, DE et al (1983, J Exp Med 158:1211).

In order to provide a basis for diagnosis, normal or standard values for DBIH expression
30 must be established. This is accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with antibody to DBIH under conditions suitable for complex formation which are well known in the art. The amount of standard complex formation

may be quantified by comparing various artificial membranes containing known quantities of DBIH with both control and disease samples from biopsied tissues. Then, standard values obtained from normal samples may be compared with values obtained from samples from subjects potentially affected by disease. Deviation between standard and subject values
5 establishes the presence of disease state.

DRUG SCREENING

DBIH, its catalytic or immunogenic fragments or oligopeptides thereof, can be used for screening therapeutic compounds in any of a variety of drug screening techniques. The fragment employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface,
10 or located intracellularly. The formation of binding complexes, between DBIH and the agent being tested, may be measured.

Another technique for drug screening which may be used for high throughput screening of compounds having suitable binding affinity to the DBIH is described in detail in "Determination of Amino Acid Sequence Antigenicity" by Geysen HN, WO Application 84/03564, published on
15 September 13, 1984, and incorporated herein by reference. In summary, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with fragments of DBIH and washed. Bound DBIH is then detected by methods well known in the art. Substantially purified DBIH can also be coated directly onto plates for use in the aforementioned drug screening
20 techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding DBIH specifically compete with a test compound for binding DBIH. In this manner, the antibodies can be used to detect the presence of any peptide
25 which shares one or more antigenic determinants with DBIH.

USES OF THE POLYNUCLEOTIDE ENCODING DBIH

A polynucleotide encoding DBIH, or any part thereof, may be used for diagnostic and/or therapeutic purposes. For diagnostic purposes, the DBIH of this invention may be used to detect and quantitate gene expression in biopsied tissues in which expression of DBIH may be
30 implicated. The diagnostic assay is useful to distinguish between absence, presence, and excess expression of DBIH and to monitor regulation of DBIH levels during therapeutic intervention. Included in the scope of the invention are oligonucleotide sequences, antisense RNA and DNA

molecules, and PNAs.

Another aspect of the subject invention is to provide for hybridization or PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding DBIH or closely related molecules. The specificity of the probe, whether it is made from a highly specific region, eg, 10 unique nucleotides in the 5' regulatory region, or a less specific region, eg, especially in the 3' region, and the stringency of the hybridization or amplification (maximal, high, intermediate or low) will determine whether the probe identifies only naturally occurring DBIH, alleles or related sequences.

Probes may also be used for the detection of related sequences and should preferably contain at least 50% of the nucleotides from any of these DBIH encoding sequences. The hybridization probes of the subject invention may be derived from the nucleotide sequence of SEQ ID NO:2 or from genomic sequence including promoter, enhancer elements and introns of the naturally occurring DBIH. Hybridization probes may be labeled by a variety of reporter groups, including radionuclides such as ^{32}P or ^{35}S , or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

Other means for producing specific hybridization probes for DBIH DNAs include the cloning of nucleic acid sequences encoding DBIH or DBIH derivatives into vectors for the production of mRNA probes. Such vectors are known in the art and are commercially available and may be used to synthesize RNA probes *in vitro* by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriate radioactively labeled nucleotides.

Diagnostics

Polynucleotide sequences encoding DBIH may be used for the diagnosis of conditions or diseases with which the expression of DBIH is associated. For example, polynucleotide sequences encoding DBIH may be used in hybridization or PCR assays of fluids or tissues from biopsies to detect DBIH expression. The form of such qualitative or quantitative methods may include Southern or northern analysis, dot blot or other membrane-based technologies; PCR technologies; dip stick, pin, chip and ELISA technologies. All of these techniques are well known in the art and are the basis of many commercially available diagnostic kits.

The DBIH nucleotide sequence disclosed herein provide the basis for assays that detect activation or induction associated with inflammation or disease. The DBIH nucleotide sequence may be labeled by methods known in the art and added to a fluid or tissue sample from a patient

under conditions suitable for the formation of hybridization complexes. After an incubation period, the sample is washed with a compatible fluid which optionally contains a dye (or other label requiring a developer) if the nucleotide has been labeled with an enzyme. After the compatible fluid is rinsed off, the dye is quantitated and compared with a standard. If the amount
5 of dye in the biopsied or extracted sample is significantly elevated over that of a comparable control sample, the nucleotide sequence has hybridized with nucleotide sequences in the sample, and the presence of elevated levels of DBIH nucleotide sequences in the sample indicates the presence of the associated inflammation and/or disease.

Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment
10 regime in animal studies, in clinical trials, or in monitoring the treatment of an individual patient. In order to provide a basis for the diagnosis of disease, a normal or standard profile for DBIH expression must be established. This is accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with DBIH, or a portion thereof, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified
15 by comparing the values obtained for normal subjects with a dilution series of DBIH run in the same experiment where a known amount of substantially purified DBIH is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients afflicted with DBIH-associated diseases. Deviation between standard and subject values is used to establish the presence of disease.

20 Once disease is established, a therapeutic agent is administered and a treatment profile is generated. Such assays may be repeated on a regular basis to evaluate whether the values in the profile progress toward or return to the normal or standard pattern. Successive treatment profiles may be used to show the efficacy of treatment over a period of several days or several months.

Polymerase Chain Reaction (PCR) as described in US Patent Nos. 4,683,195 and
25 4,965,188 provides additional uses for oligonucleotides based upon the DBIH sequence. Such oligomers are generally chemically synthesized, but they may be generated enzymatically or produced from a recombinant source. Oligomers generally comprise two nucleotide sequences, one with sense orientation (5'→3') and one with antisense (3'←5'), employed under optimized conditions for identification of a specific gene or condition. The same two oligomers, nested sets
30 of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantitation of closely related DNA or RNA sequences.

Additionally, methods which may be used to quantitate the expression of a particular

Therapeutic Use

Expression vectors derived from retroviruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids, may be used for delivery of nucleotide sequences to the targeted organ, tissue or cell population. Methods which are well known to those skilled in the art can be used to construct recombinant vectors which will express antisense DBIH. See, for example, the techniques described in Sambrook et al (supra) and Ausubel et al (supra).

Genes encoding DBIH can be turned off by transfecting a cell or tissue with expression vectors which express high levels of a desired DBIH fragment. Such constructs can flood cells with untranslatable sense or antisense sequences. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA molecules until all copies are disabled by endogenous nucleases. Transient expression may last for a month or more with a non-replicating

vector (Mettler I, personal communication) and even longer if appropriate replication elements are part of the vector system.

As mentioned above, modifications of gene expression can be obtained by designing antisense molecules, DNA, RNA or PNA, to the control regions of DBIH, ie, the promoters, 5 enhancers, and introns. Oligonucleotides derived from the transcription initiation site, eg, between -10 and +10 regions of the leader sequence, are preferred. The antisense molecules may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing compromises the ability of the double helix to open sufficiently for the 10 binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic advances using triplex DNA were reviewed by Gee JE et al (In: Huber BE and BI Carr (1994) Molecular and Immunologic Approaches, Futura Publishing Co, Mt Kisco NY).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the 15 ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of RNA encoding DBIH.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following 20 sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

25 Antisense molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding DBIH. Such DNA sequences may be incorporated into a wide variety of 30 vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept is inherent in the production of
5 PNAs and can be extended in all of these molecules by the inclusion of nontraditional bases such as inosine, queosine and wybutosine as well as acetyl-, methyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

Methods for introducing vectors into cells or tissues include those methods discussed
10 *infra* and which are equally suitable for *in vivo*, *in vitro* and *ex vivo* therapy. For *ex vivo* therapy, vectors are introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient is presented in US Patent Nos. 5,399,493 and 5,437,994, disclosed herein by reference. Delivery by transfection and by liposome are quite well known in the art.

15 Furthermore, the nucleotide sequences for DBIH disclosed herein may be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including but not limited to such properties as the triplet genetic code and specific base pair interactions.

DETECTION AND MAPPING OF RELATED POLYNUCLEOTIDE SEQUENCES

20 The nucleic acid sequence for DBIH can also be used to generate hybridization probes for mapping the naturally occurring genomic sequence. The sequence may be mapped to a particular chromosome or to a specific region of the chromosome using well known techniques. These include *in situ* hybridization to chromosomal spreads, flow-sorted chromosomal preparations, or artificial chromosome constructions such as yeast artificial chromosomes, bacterial artificial
25 chromosomes, bacterial P1 constructions or single chromosome cDNA libraries as reviewed in Price CM (1993; Blood Rev 7:127-34) and Trask BJ (1991; Trends Genet 7:149-54).

The technique of fluorescent *in situ* hybridization of chromosome spreads has been described, among other places, in Verma et al (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York NY. Fluorescent *in situ* hybridization of chromosomal
30 preparations and other physical chromosome mapping techniques may be correlated with additional genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of Science (265:1981f). Correlation between the location of a DBIH on a physical

chromosomal map and a specific disease (or predisposition to a specific disease) may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention may be used to detect differences in gene sequences between normal, carrier or affected individuals.

- 5 In situ hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers may be used for extending genetic maps. For example, an STS based map of the human genome was recently published by the Whitehead-MIT Center for Genomic Research (Hudson TJ et al (1995) Science 270:1945-1954). Often the placement of a gene on the chromosome of another mammalian species such as mouse
- 10 (Whitehead Institute/MIT Center for Genome Research, Genetic Map of the Mouse, Database Release 10, April 28, 1995) may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once a
- 15 disease or syndrome, such as ataxia telangiectasia (AT), has been crudely localized by genetic linkage to a particular genomic region, for example, AT to 11q22-23 (Gatti et al (1988) Nature 336:577-580), any sequences mapping to that area may represent associated or regulatory genes for further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc. among
- 20 normal, carrier or affected individuals.

PHARMACEUTICAL COMPOSITIONS

- The present invention relates to pharmaceutical compositions which may comprise nucleotides, proteins, antibodies, agonists, antagonists, or inhibitors, alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any
- 25 sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. Any of these molecules can be administered to a patient alone, or in combination with other agents, drugs or hormones, in pharmaceutical compositions where it is mixed with excipient(s) or pharmaceutically acceptable carriers. In one embodiment of the present invention, the pharmaceutically acceptable carrier is pharmaceutically inert.

30 Administration of Pharmaceutical Compositions

Administration of pharmaceutical compositions is accomplished orally or parenterally. Methods of parenteral delivery include topical, intra-arterial (directly to the tumor),

intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraperitoneal, or intranasal administration. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into
5 preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of "Remington's Pharmaceutical Sciences" (Maack Publishing Co, Easton PA).

Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral
10 administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the
15 mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; and gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired,
20 disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores are provided with suitable coatings such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent
25 mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, ie, dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders such as lactose or
30 starches, lubricants such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations for parenteral administration include aqueous solutions of active compounds. For injection, the pharmaceutical compositions of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiologically buffered saline. Aqueous injection
5 suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Optionally, the suspension may also contain suitable
10 stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Manufacture and Storage

15 The pharmaceutical compositions of the present invention may be manufactured in a manner that known in the art, eg, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic,
20 etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder in 1 mM-50 mM histidine, 0.1%-2% sucrose, 2%-7% mannitol at a pH range of 4.5 to 5.5 that is combined with buffer prior to use.

After pharmaceutical compositions comprising a compound of the invention formulated
25 in a acceptable carrier have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. For administration of DBIH, such labeling would include amount, frequency and method of administration.

Therapeutically Effective Dose

Pharmaceutical compositions suitable for use in the present invention include
30 compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

A therapeutically effective dose refers to that amount of protein or its antibodies, antagonists, or inhibitors which ameliorate the symptoms or condition. Therapeutic efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, eg, ED₅₀ (the dose therapeutically effective in 50% of the population) and LD₅₀ (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD₅₀/ED₅₀. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage is chosen by the individual physician in view of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Additional factors which may be taken into account include the severity of the disease state, eg, tumor size and location; age, weight and gender of the patient; diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long acting pharmaceutical compositions might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature. See US Patent Nos. 4,657,760; 5,206,344; or 5,225,212. Those skilled in the art will employ different formulations for nucleotides than for 30 proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

The examples below are provided to illustrate the subject invention and are not included

for the purpose of limiting the invention.

INDUSTRIAL APPLICABILITY

I cDNA Library Construction

The normal tissue used for paraganglion cDNA library construction was obtained from a
5 46 year-old male (Lot #0084 ; Mayo Clinic, Rochester MN). The frozen tissue was homogenized
and lysed in guanidinium isothiocyanate solution using a Brinkmann Homogenizer Polytron PT-
3000 (Brinkmann Instruments, Westbury NJ). The lysate was centrifuged over a 5.7 M CsCl
cushion using an Beckman SW28 rotor in a Beckman L8-70M Ultracentrifuge (Beckman
Instruments) for 18 hours at 25,000 rpm at ambient temperature. The RNA was extracted twice
10 with acid phenol pH 4.0 following Stratagene's RNA isolation protocol and precipitated using 0.3
M sodium acetate and 2.5 volumes of ethanol, resuspended in DEPC-treated water and DNase
treated for 15 min at 37°C. The reaction was stopped with an equal volume of acid phenol and
the RNA was isolated using the Qiagen Oligotex kit (QIAGEN Inc, Chatsworth CA) and used to
construct the cDNA library.

15 The RNA was handled according to the recommended protocols in the SuperScript
Plasmid System for cDNA Synthesis and Plasmid Cloning (catalog #18248-013; Gibco/BRL),
and cDNAs were ligated into pSport I. The plasmid pSport I was subsequently transformed into
DH5a™ competent cells (Cat. #18258-012, Gibco/BRL).

II Isolation and Sequencing of cDNA Clones

20 Plasmid DNA was released from the cells and purified using the Miniprep Kit (Catalogue
77468; Advanced Genetic Technologies Corporation, Gaithersburg MD). This kit consists of a
96 well block with reagents for 960 purifications. The recommended protocol was employed
except for the following changes: 1) the 96 wells were each filled with only 1 ml of sterile
Terrific Broth (Catalog # 22711, LIFE TECHNOLOGIES™, Gaithersburg MD) with
25 carbenicillin at 25 mg/L and glycerol at 0.4%; 2) the bacteria were cultured for 24 hours after the
wells were inoculated and then lysed with 60 µl of lysis buffer; 3) a centrifugation step
employing the Beckman GS-6R @2900 rpm for 5 min was performed before the contents of the
block were added to the primary filter plate; and 4) the optional step of adding isopropanol to
TRIS buffer was not routinely performed. After the last step in the protocol, samples were
30 transferred to a Beckman 96-well block for storage.

Alternative methods of purifying plasmid DNA include the use of MAGIC
MINIPREP™ DNA Purification System (Catalogue #A7100, Promega, Madison WI) or

QIAwell™-8 Plasmid, QIAwell PLUS DNA and QIAwell ULTRA DNA Purification Systems (QIAGEN® Chatsworth CA).

The cDNAs were sequenced by the method of Sanger F and AR Coulson (1975; J Mol Biol 94:441f), using a Hamilton Micro Lab 2200 (Hamilton, Reno NV) in combination with four
5 Peltier Thermal Cyclers (PTC200 from MJ Research, Watertown MA) and Applied Biosystems 377 or 373 DNA Sequencing Systems (Perkin Elmer), and the reading frame was determined.

III Homology Searching of cDNA Clones and Their Deduced Proteins

Each cDNA was compared to sequences in GenBank using a search algorithm developed by Applied Biosystems and incorporated into the INHERIT- 670 Sequence Analysis System. In
10 this algorithm, Pattern Specification Language (TRW Inc. Los Angeles CA) was used to determine regions of homology. The three parameters that determine how the sequence comparisons run were window size, window offset, and error tolerance. Using a combination of these three parameters, the DNA database was searched for sequences containing regions of homology to the query sequence, and the appropriate sequences were scored with an initial value.
15 Subsequently, these homologous regions were examined using dot matrix homology plots to distinguish regions of homology from chance matches. Smith-Waterman alignments were used to display the results of the homology search.

Peptide and protein sequence homologies were ascertained using the INHERIT™ 670 Sequence Analysis System in a way similar to that used in DNA sequence homologies. Pattern
20 Specification Language and parameter windows were used to search protein databases for sequences containing regions of homology which were scored with an initial value. Dot-matrix homology plots were examined to distinguish regions of significant homology from chance matches.

BLAST, which stands for Basic Local Alignment Search Tool (Altschul SF (1993) J Mol
25 Evol 36:290-300; Altschul, SF et al (1990) J Mol Biol 215:403-10), was used to search for local sequence alignments. BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs. BLAST is useful for matches which do not contain gaps. The fundamental unit of BLAST algorithm output is the
30 High-scoring Segment Pair (HSP).

An HSP consists of two sequence fragments of arbitrary but equal lengths whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold or

cutoff score set by the user. The BLAST approach is to look for HSPs between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. The parameter E establishes the statistically significant threshold for reporting database sequence matches. E is interpreted as the upper bound of the expected frequency of chance occurrence of an HSP (or set of HSPs) within the context of the entire database search. Any database sequence whose match satisfies E is reported in the program output.

IV Northern Analysis

Northern analysis is a laboratory technique used to detect the presence of a transcript of a gene and involves the hybridization of a labeled nucleotide sequence to a membrane on which RNAs from a particular cell type or tissue have been bound (Sambrook et al. supra).

Analogous computer techniques use BLAST (Altschul SF 1993 and 1990, supra) to search for identical or related molecules in nucleotide databases such as GenBank or the LIFESEQ™ database (Incyte, Palo Alto CA). This analysis is much faster than multiple, membrane-based hybridizations. In addition, the sensitivity of the computer search can be modified to determine whether any particular match is categorized as exact or homologous.

The basis of the search is the product score which is defined as:

$$\frac{\% \text{ sequence identity} \times \% \text{ maximum BLAST score}}{100}$$

100

and it takes into account both the degree of similarity between two sequences and the length of the sequence match. For example, with a product score of 40, the match will be exact within a 1-2% error; and at 70, the match will be exact. Homologous molecules are usually identified by selecting those which show product scores between 15 and 40, although lower scores may identify related molecules.

V Extension of DBIH to Full Length or to Recover Regulatory Elements

The nucleic acid sequence encoding full length DBIH (SEQ ID NO:2) is used to design oligonucleotide primers for extending a partial nucleotide sequence to full length or for obtaining 5' sequences from genomic libraries. One primer is synthesized to initiate extension in the antisense direction (XLR) and the other is synthesized to extend sequence in the sense direction (XLF). Primers allow the extension of the known DBIH nucleotide sequence "outward" generating amplicons containing new, unknown nucleotide sequence for the region of interest (US Patent Application 08/487,112, filed June 7, 1995, specifically incorporated by reference).

The initial primers are designed from the cDNA using OLIGO® 4.06 Primer Analysis Software (National Biosciences), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72° C. Any stretch of nucleotides which would result in hairpin structures and primer-
5 primer dimerizations is avoided.

The original, selected cDNA libraries, or a human genomic library are used to extend the sequence; the latter is most useful to obtain 5' upstream regions. If more extension is necessary or desired, additional sets of primers are designed to further extend the known region.

By following the instructions for the XL-PCR kit (Perkin Elmer) and thoroughly mixing
10 the enzyme and reaction mix, high fidelity amplification is obtained. Beginning with 40 pmol of each primer and the recommended concentrations of all other components of the kit, PCR is performed using the Peltier Thermal Cycler (PTC200; MJ Research, Watertown MA) and the following parameters:

	Step 1	94° C for 1 min (initial denaturation)
15	Step 2	65° C for 1 min
	Step 3	68° C for 6 min
	Step 4	94° C for 15 sec
	Step 5	65° C for 1 min
	Step 6	68° C for 7 min
20	Step 7	Repeat step 4-6 for 15 additional cycles
	Step 8	94° C for 15 sec
	Step 9	65° C for 1 min
	Step 10	68° C for 7:15 min
	Step 11	Repeat step 8-10 for 12 cycles
25	Step 12	72° C for 8 min
	Step 13	4° C (and holding)

A 5-10 μ l aliquot of the reaction mixture is analyzed by electrophoresis on a low concentration (about 0.6-0.8%) agarose mini-gel to determine which reactions were successful in
30 extending the sequence. Bands thought to contain the largest products were selected and cut out of the gel. Further purification involves using a commercial gel extraction method such as QIAQuick™ (QIAGEN Inc). After recovery of the DNA, Klenow enzyme was used to trim single-stranded, nucleotide overhangs creating blunt ends which facilitate religation and cloning.

After ethanol precipitation, the products are redissolved in 13 μ l of ligation buffer, 1 μ l
35 T4-DNA ligase (15 units) and 1 μ l T4 polynucleotide kinase are added, and the mixture is incubated at room temperature for 2-3 hours or overnight at 16° C. Competent *E. coli* cells (in 40 μ l of appropriate media) are transformed with 3 μ l of ligation mixture and cultured in 80 μ l of

SOC medium (Sambrook J et al, supra). After incubation for one hour at 37° C, the whole transformation mixture is plated on Luria Bertani (LB)-agar (Sambrook J et al, supra) containing 2xCarb. The following day, several colonies are randomly picked from each plate and cultured in 150 µl of liquid LB/2xCarb medium placed in an individual well of an appropriate, 5 commercially-available, sterile 96-well microtiter plate. The following day, 5 µl of each overnight culture is transferred into a non-sterile 96-well plate and after dilution 1:10 with water, 5 µl of each sample is transferred into a PCR array.

For PCR amplification, 18 µl of concentrated PCR reaction mix (3.3x) containing 4 units of rTth DNA polymerase, a vector primer and one or both of the gene specific primers used for 10 the extension reaction are added to each well. Amplification is performed using the following conditions:

	Step 1	94° C for 60 sec
	Step 2	94° C for 20 sec
	Step 3	55° C for 30 sec
15	Step 4	72° C for 90 sec
	Step 5	Repeat steps 2-4 for an additional 29 cycles
	Step 6	72° C for 180 sec
	Step 7	4° C (and holding)

Aliquots of the PCR reactions are run on agarose gels together with molecular weight 20 markers. The sizes of the PCR products are compared to the original partial cDNAs, and appropriate clones are selected, ligated into plasmid and sequenced.

VI Labeling and Use of Hybridization Probes

Hybridization probes derived from SEQ ID NO:2 are employed to screen cDNAs, genomic DNAs or mRNAs. Although the labeling of oligonucleotides, consisting of about 20 25 base-pairs, is specifically described, essentially the same procedure is used with larger cDNA fragments. Oligonucleotides are designed using state-of-the-art software such as OLIGO 4.06 (National Biosciences), labeled by combining 50 pmol of each oligomer and 250 mCi of [γ -³²P] adenosine triphosphate (Amersham, Chicago IL) and T4 polynucleotide kinase (DuPont NEN®, Boston MA). The labeled oligonucleotides are substantially purified with Sephadex G-25 super 30 fine resin column (Pharmacia). A portion containing 10⁷ counts per minute of each of the sense and antisense oligonucleotides is used in a typical membrane based hybridization analysis of human genomic DNA digested with one of the following endonucleases (Ase I, Bgl II, Eco RI, Pst I, Xba I, or Pvu II; DuPont NEN®).

The DNA from each digest is fractionated on a 0.7 percent agarose gel and transferred to

nylon membranes (Nytran Plus, Schleicher & Schuell, Durham NH). Hybridization is carried out for 16 hours at 40°C. To remove nonspecific signals, blots are sequentially washed at room temperature under increasingly stringent conditions up to 0.1 x saline sodium citrate and 0.5% sodium dodecyl sulfate. After XOMAT AR™ film (Kodak, Rochester NY) is exposed to the
5 blots in a Phosphorimager cassette (Molecular Dynamics, Sunnyvale CA) for several hours, hybridization patterns are compared visually.

VII Antisense Molecules

The nucleotide sequence encoding DBIH, or any part thereof, is used to inhibit in vivo or in vitro expression of naturally occurring DBIH. Although use of antisense oligonucleotides,
10 comprising about 20 base-pairs, is specifically described, essentially the same procedure is used with larger cDNA fragments. An oligonucleotide based on the coding sequence of DBIH as shown in Figures 1A, 1B and 1C is used to inhibit expression of naturally occurring DBIH. The complementary oligonucleotide is designed from the most unique 5' sequence as shown in
15 Figures 1A, 1B and 1C and used either to inhibit transcription by preventing promoter binding to the upstream nontranslated sequence or translation of an DBIH transcript by preventing the ribosome from binding. Using an appropriate portion of the leader and 5' sequence of SEQ ID NO:2, an effective antisense oligonucleotide includes any 15-20 nucleotides spanning the region which translates into the signal or early coding sequence of the polypeptide as shown in Figures 1A, 1B and 1C.

20 VIII Expression of DBIH

Expression of DBIH is accomplished by subcloning the cDNAs into appropriate vectors and transfecting the vectors into host cells. In this case, the cloning vector, pSport, previously used for the generation of the cDNA library is used to express DBIH in E. coli. Upstream of the cloning site, this vector contains a promoter for β -galactosidase, followed by sequence containing
25 the amino-terminal Met and the subsequent 7 residues of β -galactosidase. Immediately following these eight residues is a bacteriophage promoter useful for transcription and a linker containing a number of unique restriction sites.

Induction of an isolated, transfected bacterial strain with IPTG using standard methods produces a fusion protein which consists of the first seven residues of β -galactosidase, about 5 to
30 15 residues of linker, and the full length DBIH. The signal sequence directs the secretion of DBIH into the bacterial growth media which can be used directly in the following assay for activity.

IX DBIH Activity

The binding of a ligand to DBIH is assayed by monitoring the resulting changes in enthalpy (heat production or absorption) in an isothermal titration microcalorimeter (Micro-Cal Inc, Northampton MA). Titration microcalorimetry measurements do not require labeling of the
5 ligand or receptor molecules; detection is based solely on the intrinsic change in the heat of enthalpy upon binding. Multiple computer-controlled injections of a known volume of ligand solution are directed into a thermally-controlled chamber containing DBIH. The change in enthalpy after each injection is plotted against the number of injections, producing a binding isotherm. The volumes and concentrations of the injected ligand and of the DBIH solution are
10 used along with the binding isotherm to calculate values for the number, affinity, and association of DBIH with the candidate ligand.

X Production of DBIH Specific Antibodies

DBIH substantially purified using PAGE electrophoresis (Sambrook, supra) is used to immunize rabbits and to produce antibodies using standard protocols. The amino acid sequence
15 translated from DBIH is analyzed using DNASTar software (DNASTar Inc) to determine regions of high immunogenicity and a corresponding oligopolypeptide is synthesized and used to raise antibodies by means known to those of skill in the art. Analysis to select appropriate epitopes, such as those near the C-terminus or in hydrophilic regions (shown in Figure 3) is described by Ausubel FM et al (supra).

20 Typically, the oligopeptides are 15 residues in length, synthesized using an Applied Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry, and coupled to keyhole limpet hemocyanin (KLH, Sigma) by reaction with M-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Ausubel FM et al, supra). Rabbits are immunized with the oligopeptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity,
25 for example, by binding the peptide to plastic, blocking with 1% BSA, reacting with rabbit antisera, washing, and reacting with radioiodinated, goat anti-rabbit IgG.

XI Purification of Naturally Occurring DBIH Using Specific Antibodies

Naturally occurring or recombinant DBIH is substantially purified by immunoaffinity chromatography using antibodies specific for DBIH. An immunoaffinity column is constructed
30 by covalently coupling DBIH antibody to an activated chromatographic resin such as CnBr-activated Sepharose (Pharmacia Biotech). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

Cellular fractions from cells containing DBIH are prepared by solubilization of the whole cell and isolation of subcellular fractions by differential centrifugation, by the addition of detergent, or by other methods well known in the art. Alternatively, soluble DBIH containing a signal sequence may be secreted in useful quantity into the medium in which the cells are grown.

5 A fractionated DBIH-containing preparation is passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of DBIH (eg, high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt antibody/DBIH binding (eg, a buffer of pH 2-3 or a high concentration of a chaotrope such as urea or thiocyanate ion), and DBIH is collected.

10 XII Identification of Molecules Which Interact with DBIH

DBIH is useful as a research tools for identification, characterization and purification of molecules with which it interacts. In one embodiment of affinity purification, DBIH is covalently coupled to a chromatography column. Cells and their membranes are extracted, endogenous DBIH is removed and various DBIH-free subcomponents are passed over the
15 column. DBIH-associated molecules bind to the column by virtue of their biological affinity. The DBIH-complex is recovered from the column, dissociated and the recovered molecule is subjected to either N-terminal protein sequencing or to high-performance liquid chromatography/mass spectrometry (HPLC/MS). This amino acid sequence or mass spectral analysis is then used to identify the captured molecule or, in the case of a protein ligand, to
20 design degenerate oligonucleotide probes for cloning its gene from an appropriate cDNA library.

In an alternate method, monoclonal antibodies are raised against DBIH and screened to identify those compounds which inhibit the binding of the antibody to DBIH. These monoclonal antibodies are then used in affinity purification or expression cloning of associated molecules.

Other soluble binding molecules are identified in a similar manner. DBIH previously
25 labelled with ^{125}I Bolton-Hunter reagent (Bolton, AE and Hunter, WM (1973) Biochem J 133:529) is incubated with extracts or biopsied materials derived from cells or tissues such as paraganglia, rheumatoid synovium, or cerebellum. After incubation, DBIH complexes (which are larger than the size of the purified DBIH molecule) are identified by a sizing technique such as size exclusion chromatography or density gradient centrifugation and are purified by methods
30 known in the art. The soluble binding protein(s) are subjected to N-terminal sequencing or mass spectrometry to obtain information sufficient for database identification, if the soluble protein or molecule is known, or for cloning, if the soluble protein is unknown.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred
5 embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

(1) GENERAL INFORMATION

- (i) APPLICANT: INCYTE PHARMACEUTICALS, INC.
- (ii) TITLE OF THE INVENTION: NOVEL HUMAN DBI/ACBP-LIKE PROTEIN
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Incyte Pharmaceuticals, Inc.
 - (B) STREET: 3174 Porter Drive
 - (C) CITY: Palo Alto
 - (D) STATE: CA
 - (E) COUNTRY: U.S.
 - (F) ZIP: 94304
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette
 - (B) COMPUTER: IBM Compatible
 - (C) OPERATING SYSTEM: DOS
 - (D) SOFTWARE: FastSEQ Version 1.5
- (vi) CURRENT APPLICATION DATA:
 - (A) PCT APPLICATION NUMBER: To Be Assigned
 - (B) FILING DATE: Herewith
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/700,626
 - (B) FILING DATE: 16-AUG-1996
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Billings, Lucy J.
 - (B) REGISTRATION NUMBER: 36,749
 - (C) REFERENCE/DOCKET NUMBER: PF-0115 PCT
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 415-855-0555
 - (B) TELEFAX: 415-845-4166

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 145 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
 (A) LIBRARY: PGANNOT01
 (B) CLONE: 620984

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Met Ala Ser Ser Phe Leu Pro Ala Gly Ala Ile Thr Gly Asp Ser Gly
 1           5           10           15
Gly Glu Leu Ser Ser Gly Asp Asp Ser Gly Glu Val Glu Phe Pro His
 20           25           30
Ser Pro Glu Ile Glu Glu Thr Ser Cys Leu Ala Glu Leu Phe Glu Lys
 35           40           45
Ala Ala Ala His Leu Gln Gly Leu Ile Gln Val Ala Ser Arg Glu Gln
 50           55           60
Leu Leu Tyr Leu Tyr Ala Arg Tyr Lys Gln Val Lys Val Gly Asn Cys
 65           70           75           80
Asn Thr Pro Lys Pro Ser Phe Phe Asp Phe Glu Gly Lys Gln Lys Trp
 85           90           95
Glu Ala Trp Lys Ala Leu Gly Asp Ser Ser Pro Ser Gln Ala Met Gln
 100          105          110
Glu Tyr Ile Ala Val Val Lys Lys Leu Asp Pro Gly Trp Asn Pro Gln
 115          120          125
Ile Pro Glu Lys Lys Arg Lys Arg Ser Lys Tyr Lys Val Trp Ala Ser
 130          135          140
Tyr
145

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1123 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: PGANNOT01
- (B) CLONE: 620984

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

TTGGGTCCGA CTGGAGCTCA GGCTCGCGAC CCAGACTGGT GGGCCAGGCC TCCAAGCCGG 60
CCTTACACCC AATCCAAGGA GGACAGACCG GACACAGAGG GACGGAGCGA GCAAGGAGAC 120
ATGGCTTCAT CATTCTGCTC CGCGGGGGCC ATCACC GGCG ACAGCGGTGG AGAGCTGAGC 180
TCAGGGGACG ACTCCGGGGA GGTGGAGTTC CCCCATAGCC CTGAGATCGA GGAGACCACT 240
TGCCTGGCCG AGCTGTTTGA GAAGGCTGCC GCGCACCTGC AAGGCCTGAT TCAGGTGGCC 300
AGCAGGGAGC AGCTCTTGTA CCTGTATGCC AGGTACAAAC AGGTCAAAGT TGGAAATTGT 360
AATACTCCTA AACCAAGCTT CTTTGATTTT GAAGGAAAGC AAAAATGGGA AGCTTGGA 420
GCACTTGGTG ATTCAAGCCC CAGCCAAGCA ATGCAGGAAT ATATCGCAGT AGTTAAAA 480
CTAGATCCAG GTTGAATCC TCAGATACCA GAGAAGAAAC GGAAAAGAAG CAAATACA 540
GTTTGGGCCA GTTATTAGTT CTCTATATCA TGAAGAAACC ATCAGGGAAG AGACAAAA 600
ATATTTGATT ACTGCAGGGA AAACAACATT GACCATATAA CCAAAGCCAT CAAATCGA 660
AATGTGGATG TGAATGTGAA AGATGAAGAG GGTAGGGCTC TACTTCACTG GGCCTGTG 720
CGAGGACATA AGGAACTAGT CACAGTGTG CTGCAACATA GAGCTGACAT TAACGTG 780
GACAATGAAG GCCAAACAGC TCTACATTAT GCCTCTGCCT GTGAGTTTCT GGATATTG 840
GAGCTGCTGC TCCAGTCTGG TGCTGACCCC ACTCTCCGAG ACCAGGATGG CTGCCTGC 900
GAGGAGGTGA CAGGCTGCAA AACAGTTTCT TTGGTGCTGC AGCGGCACAC AACTGGCA 960
GCTTAATCAA AAGACTGGAA AACTGCAGTC TGTAATAGCA TAAGGCTTCC ATTATGAA 1020

```

AAACTACAA AAATAATACT TCTTTCCAC CCGTCTTTGG TATGTATTGG CTAATAAAAT 1080
CAGTTCTGTG GAACTGGGAA AAAAAAAAAA AAAAAAAAAA AAA 1123

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 104 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: GenBank
- (B) CLONE: 181478

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Trp Gly Asp Leu Trp Leu Leu Pro Pro Ala Ser Ala Asn Pro Gly
1 5 10 15
Thr Gly Thr Glu Ala Glu Phe Glu Lys Ala Ala Glu Glu Val Arg His
20 25 30
Leu Lys Thr Lys Pro Ser Asp Glu Glu Met Leu Phe Ile Tyr Gly His
35 40 45
Tyr Lys Gln Ala Thr Val Gly Asp Ile Asn Thr Glu Arg Pro Gly Met
50 55 60
Leu Asp Phe Thr Gly Lys Ala Lys Trp Asp Ala Trp Asn Glu Leu Lys
65 70 75 80
Gly Thr Ser Lys Glu Asp Ala Met Lys Ala Tyr Ile Asn Lys Val Glu
85 90 95
Glu Leu Lys Lys Lys Tyr Gly Ile
100

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 86 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: GenBank
- (B) CLONE: 118276

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Gln Ala Glu Phe Glu Lys Ala Ala Glu Glu Val Arg His Leu Lys
1 5 10 15
Thr Lys Pro Ser Asp Glu Glu Met Leu Phe Ile Tyr Gly His Tyr Lys
20 25 30
Gln Ala Thr Val Gly Asp Ile Asn Thr Glu Arg Pro Gly Met Leu Asp
35 40 45

Phe Thr Gly Lys Ala Lys Trp Asp Ala Trp Asn Glu Leu Lys Gly Thr
 50 55 60
 Ser Lys Glu Asp Ala Met Lys Ala Tyr Ile Asn Lys Val Glu Glu Leu
 65 70 75 80
 Lys Lys Lys Tyr Gly Ile
 85

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 86 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: GenBank
- (B) CLONE: 118275

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Gln Ala Glu Phe Asp Lys Ala Ala Glu Glu Val Lys His Leu Lys
 1 5 10 15
 Thr Lys Pro Ala Asp Glu Glu Met Leu Phe Ile Tyr Ser His Tyr Lys
 20 25 30
 Gln Ala Thr Val Gly Asp Ile Asn Thr Glu Arg Pro Gly Met Leu Asp
 35 40 45
 Phe Lys Gly Lys Ala Lys Trp Asp Ala Trp Asn Glu Leu Lys Gly Thr
 50 55 60
 Ser Lys Glu Asp Ala Met Lys Ala Tyr Ile Asp Lys Val Glu Glu Leu
 65 70 75 80
 Lys Lys Lys Tyr Gly Ile
 85

CLAIMS

1. A substantially purified polypeptide comprising the amino acid sequence of SEQ ID NO:1 or fragments thereof.
2. An isolated and purified polynucleotide sequence encoding the polypeptide of claim 1.
- 5 3. An isolated and purified polynucleotide sequence of claim 3 consisting of the sequence of SEQ ID NO:2 or variants thereof.
4. A polynucleotide sequence comprising the complement of SEQ ID NO:2 or variants thereof.
5. An isolated polynucleotide sequence hybridizes under stringent conditions to SEQ ID
10 NO:2.
6. A recombinant expression vector containing a polynucleotide sequence of claim 3.
7. A recombinant host cell comprising a polynucleotide sequence of claim 3.
8. A method for producing the polypeptide consisting of SEQ ID NO:1 or fragments thereof, the method comprising the steps of:
15 a) culturing the host cell of claim 7 under conditions suitable for the expression of the polypeptide; and
b) recovering the polypeptide from the host cell culture.
9. A purified antibody which binds specifically to the polypeptide of claim 1.
10. A pharmaceutical composition comprising the polypeptide of SEQ ID NO:1 in
20 conjunction with a suitable pharmaceutical carrier.
11. A polypeptide for drug delivery consisting of SEQ ID NO:1 in combination with a therapeutic agent.

1/7

5' TTG GGT CCG ACT GGA GCT CAG GCT CGC GAC CCA GAC TGG TGG GCC AGG CCT CCA
 9 18 27 36 45 54
 AGC CGG CCT TAC ACC CAA TCC AAG GAG GAC AGA CCG GAC ACA GAG GGA CGG AGC
 63 72 81 90 99 108
 GAG CAA GGA GAC ATG GCT TCA TCA TTC CTG CCC GCG GGG GGC ATC ACC GGC GAC
 117 126 135 144 153 162
 M A S S F L P A G A I T G D
 AGC GGT GGA GAG CTG AGC TCA GGG GAC GAC TCC GGG GAG GTG GAG TTC CCC CAT
 171 180 189 198 207 216
 S G E L S S G D D S G E V E F P H
 AGC CCT GAG ATC GAG GAG ACC AGT TGC CTG GCC GAG CTG TTT GAG AAG GCT GCC
 225 234 243 252 261 270
 S P E I E E T S C L A E L F E K A A
 GCG CAC CTG CAA GGC CTG ATT CAG GTG GCC AGC AGG GAG CAG CTC TTG TAC CTG
 279 288 297 306 315 324
 A H L Q G L I Q V A S R E Q L L Y L
 TAT GCC AGG TAC AAA CAG GTC AAA GTT GGA AAT TGT AAT ACT CCT AAA CCA AGC
 333 342 351 360 369 378
 Y A R Y K Q V K V G N C N T P K P S

FIGURE 1A

2/7

387	396	405	414	423	432
TTC TTT GAT TTT GAA GGA AAG CAA AAA TGG GAA GCT TGG AAA GCA CTT GGT GAT					
F F D F E G K Q K W E A W K A L G D					
441	450	459	468	477	486
TCA AGC CCC AGC CAA GCA ATG CAG GAA TAT ATC GCA GTA GTT AAA AAA CTA GAT					
S S P S Q A M Q E Y I A V V K K L D					
495	504	513	522	531	540
CCA GGT TGG AAT CCT CAG ATA CCA GAG AAG AAA CGG AAA AGA AGC AAA TAC AAG					
P G W N P Q I P E K K R K R S K Y K					
549	558	567	576	585	594
GTT TGG GCC AGT TAT TAG TTC TCT ATA TCA TGA AGA AAC CAT CAG GGA AGA GAC					
V W A S Y *					
603	612	621	630	639	648
AAA AAT ATA TTT GAT TAC TGC AGG GAA AAC AAC ATT GAC CAT ATA ACC AAA GCC					
657	666	675	684	693	702
ATC AAA TCG AAA AAT GTG GAT GTG AAT GTG AAA GAT GAA GAG GGT AGG GCT CTA					
711	720	729	738	747	756
CTT CAC TGG GCC TGT GAT CGA GGA CAT AAG GAA CTA GTC ACA GTG TTG CTG CAA					

FIGURE 1B

3/7

765	774	783	792	801	810
CAT AGA GCT GAC ATT AAC TGT CAG GAC AAT GAA GGC CAA ACA GCT CTA CAT TAT					
819	828	837	846	855	864
GCC TCT GCC TGT GAG TTT CTG GAT ATT GTA GAG CTG CTG CTC CAG TCT GGT GCT					
873	882	891	900	909	918
GAC CCC ACT CTC CGA GAC CAG GAT GGC TGC CTG CCA GAG GAG GTG ACA GGC TGC					
927	936	945	954	963	972
AAA ACA GTT TCT TTG GTG CTG CAG CGG CAC ACA ACT GGC AAG GCT TAA TCA AAA					
981	990	999	1008	1017	1026
GAC TGG AAA ACT GCA GTC TGT AAT AGC ATA AGG CTT CCA TTA TGA AAG AAA ACT					
1035	1044	1053	1062	1071	1080
ACA AAA ATA ATA CTT CTT TTC CAC CCG TCT TGT GTA TGT ATT GGC TAA TAA AAT					
1089	1098	1107	1116		
CAG TTC TGT GGA ACT GGG AAA AAA AAA AAA AAA AAA AAA A 3'					

FIGURE 1C

4/7

1	M A S S F	- -	L P A G A I T G D S G G E	DBIH
1	M W G D L W L	L P	P A S A N P G T G T E	GI 181478
1	- - - - -	- - - - -	- - - - - S Q	GI 118276
1	- - - - -	- - - - -	- - - - - S Q	GI 118275
19	L S S G D D S G E V E F P H S P E I E E			DBIH
21	A E - - - - -	- - - - -	- - - - -	GI 181478
3	A E - - - - -	- - - - -	- - - - -	GI 118276
3	A E - - - - -	- - - - -	- - - - -	GI 118275
39	T S C L A E L F E K A A A H L Q G L I Q			DBIH
23	- - - - -	F E K A A E E V R H L K T		GI 181478
5	- - - - -	F E K A A E E V R H L K T		GI 118276
5	- - - - -	F D K A A E E V K H L K T		GI 118275
59	V A S R E Q L L Y L Y A R Y K Q V K V G			DBIH
36	K P S D E E M L F I Y G H Y K Q A T V G			GI 181478
18	K P S D E E M L F I Y G H Y K Q A T V G			GI 118276
18	K P A D E E M L F I Y S H Y K Q A T V G			GI 118275
79	N C N T P K P S F F D F E G K Q K W E A			DBIH
56	D I N T E R P G M L D F T G K A K W D A			GI 181478
38	D I N T E R P G M L D F T G K A K W D A			GI 118276
38	D I N T E R P G M L D F K G K A K W D A			GI 118275
99	W K A L G D S S P S Q A M Q E Y I A V V			DBIH
76	W N E L K G T S K E D A M K A Y I N K V			GI 181478
58	W N E L K G T S K E D A M K A Y I N K V			GI 118276
58	W N E L K G T S K E D A M K A Y I D K V			GI 118275

FIGURE 2A

5/7

119	K K L D P G W N P Q I P E K K R K R S K	DBIH
96	E E L - - - - - - - - - - - - - - - K K K	GI 181478
78	E E L - - - - - - - - - - - - - - - K K K	GI 118276
78	E E L - - - - - - - - - - - - - - - K K K	GI 118275
139	Y K V W A S Y	DBIH
102	Y G I	GI 181478
84	Y G I	GI 118276
84	Y G I	GI 118275

FIGURE 2B

6/7

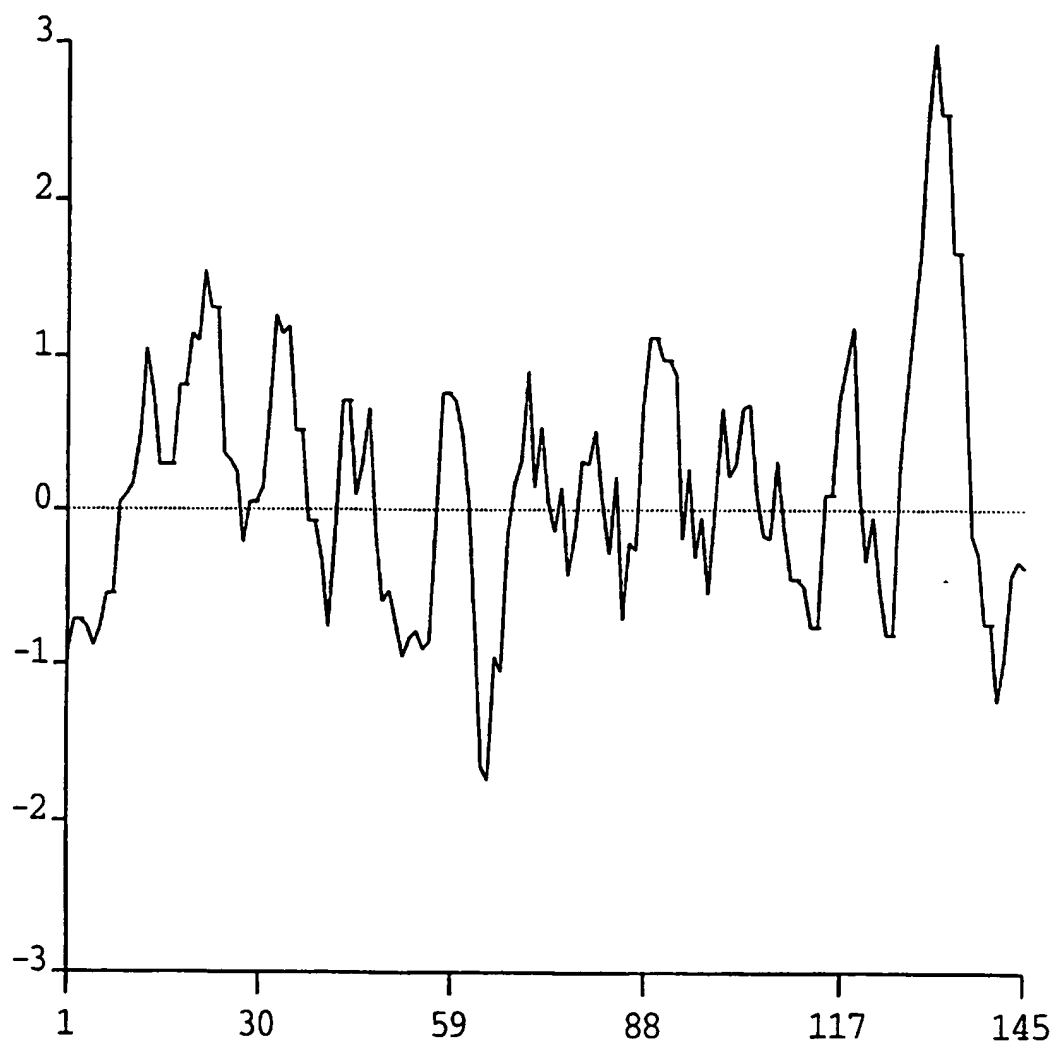


FIGURE 3

7/7

	10	20	30	40	50	60	70
MASSFLPAGAITGDSGELSSGDDSGEVEFPHSPEIEETSCLAELFEKAAHLQGLIQVASREQLLYLYA							
HELIX HHHHHh							
SHEET				ssssssss	ssssssssssssssssssssssssssss		
TURN	TTTTT	TTTTTTTTTTTTTTTT	TTTTTTT	TTTTT			
COIL		C	CC	C			

	80	90	100	110	120	130	140
RYKQVKVGNCTPKPSFFDFEGKQKWEAWKALGDSSPSQAMQEIYIAVVKKLDPGWNPQIPEKKRRRSKYK							
HELIX hhhhhh		hhhhhhhhhhhhhhhh	hhhhhhhhhhhhhh	hhhhhhhhhhhh	HHhh	h	
SHEET SSSSSS	SSSS	ssssssssssssss	ssssssssssss	ssssss		S	
TURN	TTTTTTTTTTTT	TTTTTTTT	TTTTTTTT	TTTTTTTT	TTTTTTTT	TTTTTTTT	
COIL						CC	

VWASY
HELIX hh
SHEET SSSS
TURN
COIL

FIGURE 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/14379

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/47 C12N15/70 C12N1/21 C07K16/18
A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL Database Entry MMA14318 Accession number AA014318; 3 August 1996 MARRA M, ET AL.: "The WashU-HHMI Mouse EST Project." XP002048476 see the whole document	5
X	EMBL Database Entry MMA09080; Accession number AA009080; 28 July 1996 MARRA M, ET AL.: "The WashU-HHMI Mouse EST Project." XP002048477 see the whole document	5

-/--

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

28 November 1997

Date of mailing of the international search report

19/12/1997

Name and mailing address of the ISA

European Patent Office, P.B. 5618 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Montero Lopez, B

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 97/14379

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
A	<p>SWINNEN, JOHANNES V. ET AL: "A human gene encoding diazepam - binding inhibitor / acyl - CoA - binding protein: transcription and hormonal regulation in the androgen-sensitive human prostatic adenocarcinoma cell line LNCaP" DNA CELL BIOL. (1996), 15(3), 197-208 CODEN: DCEBE8;ISSN: 1044-5498, 1996, XP002048471 see abstract see page 201, right-hand column, paragraph 2 - page 203, left-hand column, paragraph 2; figure 4 see page 205, left-hand column, paragraph 2 - page 206, right-hand column, paragraph 1</p>	1-11
A	<p>KOLMER, MEELIS ET AL: "The characterization of two diazepam binding inhibitor (DBI) transcripts in humans" BIOCHEM. J. (1995), 306(2), 327-30 CODEN: BIJOAK;ISSN: 0264-6021. 1995, XP002048472 see abstract see page 328, left-hand column, paragraph 5 - page 330, left-hand column, paragraph 1</p>	1-11
A	<p>EMBL Database entry HSACABP Accession number L76366: 5 May 1996 KROELL J.B. ET AL.: "Structure of the rat gene encoding the multifunctional acyl-CoA -binding protein: Conservation of intron 1 sequences in rodent and man" XP002048478 see the whole document</p>	1-11
A	<p>GERSUK, VIVIAN H. ET AL: "Molecular cloning and chromosomal localization of a pseudogene related to the human acyl - CoA binding protein / diazepam binding inhibitor" GENOMICS (1995), 25(2), 469-76 CODEN: GNMCEP;ISSN: 0888-7543, 1995, XP002048473 see abstract see page 470, right-hand column, paragraph 4 - page 475, left-hand column, paragraph 1</p>	1-11

1

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/14379

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MARQUARDT, HANS ET AL: "Complete amino acid sequences of bovine and human endozepines. Homology with rat diazepam - binding inhibitor"</p> <p>J. BIOL. CHEM. (1986), 261(21), 9727-31</p> <p>CODEN: JBCHA3;ISSN: 0021-9258, 1986, XP002048474</p> <p>see abstract; figure 2</p> <p>see page 9728, right-hand column, paragraph 2 - page 9729, right-hand column, paragraph 2</p> <p style="text-align: center;">---</p>	1-11
A	<p>GRAY, PATRICK W. ET AL: "Cloning and expression of cDNA for human diazepam binding inhibitor, a natural ligand of an allosteric regulatory site of the gamma.-aminobutyric acid type A receptor"</p> <p>PROC. NATL. ACAD. SCI. U. S. A. (1986), 83(19), 7547-51 CODEN: PNASA6;ISSN: 0027-8424, 1986, XP002048475</p> <p>see abstract</p> <p>see page 7547, right-hand column, paragraph 3 - page 7548, left-hand column, paragraph 3; figure 1</p> <p>see page 7549, left-hand column, paragraph 2 - page 7551, left-hand column, paragraph 2</p> <p style="text-align: center;">-----</p>	1-11